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A Constitutive Mutation of *ALK5* Disrupts Cardiac Looping and Morphogenesis in Mice[1]

Min-Ji Charng,*,†,[2] Peter A. Frenkel,*,† Qing Lin,‡ Miho Yumada,§ Robert J. Schwartz,§ Eric N. Olson,‡ Paul Overbeek,§ and Michael D. Schneider*,†,§,¶,[3]

**Molecular Cardiology Unit, †Department of Medicine, §Department of Cell Biology, and ¶Department of Molecular Physiology & Biophysics, Baylor College of Medicine, Houston, Texas 77030; and ‡Hamon Center for Basic Research in Cancer, University of Texas–Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, Texas 75235*

TGFβ family members are implicated in cardiac organogenesis, growth control, and positional information, including the direction of cardiac looping. However, genetic analysis of TGFβ signaling in mice has been confounded, in some cases, by noncardiac and generalized defects. Hence, deciphering TGFβ function in myocardium would benefit from cardiac-restricted mutations. We developed a constitutively activated type I receptor, ALK5^{L193A,P194A,T204D}, and directed it to embryonic myocardium in transgenic mice. Expression of the activated *ALK5* gene arrests looping morphogenesis and causes a linear, dilated, hypoplastic heart tube, despite normal expression of Nkx2.5 and dHAND, cardiogenic transcription factors whose absence provokes a similar phenotype. Ventricular hypoplasia was associated with precocious induction of the cyclin-dependent kinase inhibitor, p21. Thus, an ALK5-sensitive pathway mediates looping, perhaps through control of cardiac myocyte proliferation.

Key Words: **ALK5; cardiac development; looping; p21; TGFβ.**

INTRODUCTION

Cardiac organogenesis requires recruitment of precursor cells to a cardiac fate, decisions that both are accessible in *Drosophila* and are predictive for other species (Bodmer, 1993; Lilly *et al.*, 1994), but also more complex morphogenetic events that may be amenable to genetic analysis only in vertebrate models (Fishman and Chien, 1997; Olson and Srivastava, 1996). Rightward cardiac looping, the earliest left–right asymmetry in embryos, is conserved in vertebrates and involves two distinct genetic loci. The *iv* mutation, affecting an axonemal dynein, causes randomization of left–right position (Supp *et al.*, 1997); by contrast, the *inv* mutation produces consistent inversion (Yokoyama *et al.*, 1993). Molecules that have been implicated in left–right determination include transforming growth factor-β (TGFβ) family ligands and receptors—activin receptor IIA and the nodal-related factor, cNR-1, in avians (Levin *et al.*, 1995); Vg1 and Xnr-1 in *Xenopus* (Hyatt *et al.*, 1996; Lowe *et al.*, 1996); BMP4 in zebrafish (Chen *et al.*, 1997a); and lefty and nodal in mice (Lowe *et al.*, 1996; Meno *et al.*, 1996) (acting downstream from *iv* and *inv*), as well as the activin IIB receptor (Oh and Li, 1997). Finally, missense mutations in the human homologue of nodal are associated with clinical situs abnormalities (Gebbia *et al.*, 1997). At least three cardiogenic transcription factors also are required for normal looping of the heart—the homeodomain protein, Nkx2.5 (Lyons *et al.*, 1995), helix–loop–helix protein, dHAND (Srivastava *et al.*, 1997), and MADS box transcription factor, MEF2C (Lin *et al.*, 1997). Potential effector mechanisms for looping include differential recruitment, proliferation, migration, deformation, and death.

The TGFβ superfamily also is implicated in cardiac myogenesis. Decapentaplegic mediates heart formation in *Drosophila* (Frasch, 1995); bone morphogenetic proteins

[1] M.-J.C. and P.A.F. contributed equally to this work.

[2] Current address: Department of Medicine, Veterans General Hospital, Taipei, and National Yang-Ming University, Taipei, Taiwan.

[3] To whom correspondence should be addressed at Molecular Cardiology Unit, Baylor College of Medicine, One Baylor Plaza, Room 506C, Houston, TX 77030. Fax: (713)798-7437. E-mail: michaels@bcm.tmc.edu.

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(BMPs) are necessary for cardiac myogenesis in avians (Schultheiss *et al.,* 1997); and mesoderm formation itself requires nodal, BMP4, and the BMP receptor BMPR1A in mice (Hogan, 1996). In mice lacking BMP2, the heart is positioned abnormally, in the exocoelomic cavity (Zhang and Bradley, 1996). For TGFβ1, a genetic dissection of the protein's potential function in cardiac development has been impeded by early-onset inflammation, maternal (transplacental) rescue, extraembryonic defects, and potential redundancy provided by other TGFβ family members (Dickson *et al.,* 1995; Diebold *et al.,* 1995; Letterio *et al.,* 1994); however, the absence of TGFβ2 causes cardiac anomalies resembling Tetralogy of Fallot, with defects of the ventricular septum and outflow tract (Sanford *et al.,* 1997). Signal transduction for the TGFβ family entails the sequential action of two serine/threonine kinase receptors (Massagué, 1996). Type II receptors determine the specificity of ligand binding; type I receptors specify the signaling response. We previously developed a constitutively active mutation of the type I TGFβ receptor, ALK5 ($ALK5^{L193A,P194A,T204D}$), which functions autonomously in the absence of ligand and type II receptor (Charng *et al.,* 1996). The Thr mutation is sufficient to confer constitutive activity (Wieser *et al.,* 1995); the Leu-Pro mutation potentiates this ligand-independent function of the receptor, and abrogates its interaction with the immunophilin-binding protein, FKBP-12 (Charng *et al.,* 1996), a reported inhibitor of TGFβ signaling (Chen *et al.,* 1997b; Wang *et al.,* 1996). TGFβ signal transduction appears to be normal in FKBP-12-deficient mice, however (Show *et al.,* in press), implying that the greater constitutive activity of $ALK5^{L193A,P194A,T204D}$ versus $ALK5^{T204D}$ cannot be ascribed to its failure to bind FKBP-12. Features favoring the use of this activated receptor for a cardiac-restricted gain of function include its ability to signal similarly to saturating concentrations of TGFβ, to obviate the paracrine effects of secreted TGFβ, and to avoid interference by endogenous inhibitors of TGFβ itself, including decorin.

MATERIALS AND METHODS

Construction of Cardiac-Restricted, Activated ALK5 Transgenic Mice

The SV40 promoter of wild-type and mutant pALK5-HA-SV-Sport plasmids (Charng *et al.,* 1996) was replaced with the 5.5-kb α-myosin heavy chain (MHC) promoter, provided by J. Robbins (Subramaniam *et al.,* 1991). Expression cassettes were excised from the vector, purified, and injected into the pronucleus of FVB/N zygotes; injected zygotes were transferred to pseudopregnant ICR females (Taketo *et al.,* 1991). For PCR screening, the transgene (1050 bp) and endogenous β-casein (575 bp) were amplified concurrently. DNA was extracted from yolk sac and amnionic membranes. The PCR primers were: *ALK5*, forward 5′-CCA AAT GAA GAG GAC CCT TC-3′ (nucleotides 551–570), reverse 5′-CCC TAG AGG CTA GCA TAA TC-3′ (specific to the hemagglutinin epitope); *β-casein*, forward 5′-GAT GTG CTC CAG GCT AAA GTT-3′, reverse 5′-AGA AAC GGA ATG TTG TGG AGT-3′.

RT–PCR

To monitor transgene expression, RNA was purified from the heart, body, or yolk sac membranes of E10.5 embryos, treated with ribonuclease-free DNase I, annealed with oligo$(dT)_{12–18}$, and subjected to reverse transcription and PCR amplification. The *GAPDH* primers were: forward 5′-GGA TGGCCC CTC TGG AAA GC-3′; reverse 5′-GTC CTT GCT GGG GTG GGT GG-3′. To monitor endogenous gene expression, amplification was performed under conditions of linearity for each gene, using RNA from wild-type E9.5 mice, and transgene-negative littermates (−) or activated ALK5 mice (+) at E10.5. Each RNA sample was pooled from five hearts. PCR products were separated by 5% polyacrylamide gel electrophoresis and quantitated by PhosphorImager analysis (Molecular Dynamics). The gene-specific primers are: *E2F-1*, forward 5′-TGA GAC CCA ACT ACA AGC-3′ (nt 910-927), reverse 5′-CAG GTC CCC AAA GTC ACA-3′ (nt 1393–1376); *p21*, forward 5′-TCC CGT GGA CAG TGA GCA GTT G-3′ (nt 266–287), reverse 5′-GAC ACA CAG AGT GAG GGC TAA G-3′ (nt 765–744); *αMHC*, forward 5′-CTC AGC CAG GCC AAT AGA ATT-3′ (nt 4993–5012), reverse 5′-CTCATG CCC TTC ACC GAC TC-3′ (nt 5630–5611); MLC_{2V}, forward 5′-TGT TCC TCACGA TGT TTG GG-3′ (nt 281–300), reverse 5′-CTC AGT CCT TCT CTT CTC CG-3′ (nt 541–522).

In Situ Hybridization and Immunostaining

For Nkx2.5, [^{35}S]UTP-labeled RNA was synthesized from full-length Nkx2.5 cDNA in pBlueScript II SK using T7 RNA polymerase. E10.5 embryos were fixed in 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned. Sections were hybridized overnight, washed, and processed for emulsion autoradiography. Sections were poststained with Hoechst 33258 and visualized by epifluorescence and dark-field microscopy. To analyze MLC2v and dHAND by whole-mount *in situ* hybridization, embryos were fixed in 4% paraformaldehyde and probed with digoxigenin-labeled antisense RNA as described (Lin *et al.,* 1997; Srivastava *et al.,* 1997).

To delineate the expression of sarcomeric myosin heavy chains, E10.5 embryos were fixed in 10% neutral buffered Formalin and sectioned as above. Sections were treated sequentially with 3% H_2O_2 in methanol, a 1:10 dilution of normal goat serum, and mouse monoclonal MF20 antibody (Developmental Hybridoma Bank, University of Iowa) at a 1:20 dilution. Sections were washed, incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Vector) at a 1:200 dilution, developed using diaminobenzidine, and counterstained with hematoxylin. For proliferating cell nuclear antigen (PCNA), mouse monoclonal antibody to PCNA (DAKO) was used at a 1:100 dilution, followed by horseradish peroxidase-conjugated goat anti-mouse IgG as above and aminoethylcarbazole.

RESULTS

The Cardiac-Specific, Activated ALK5 Gene Causes Embryonic Lethality

Using the α-MHC promoter (Subramaniam *et al.,* 1991), activated and wild-type ALK5 transgenes were expressed in mouse myocardium (Fig. 1). Whereas litter size averaged six pups per recipient for wild-type ALK5, similar to control mice, litter size after injection with activated ALK5 ranged

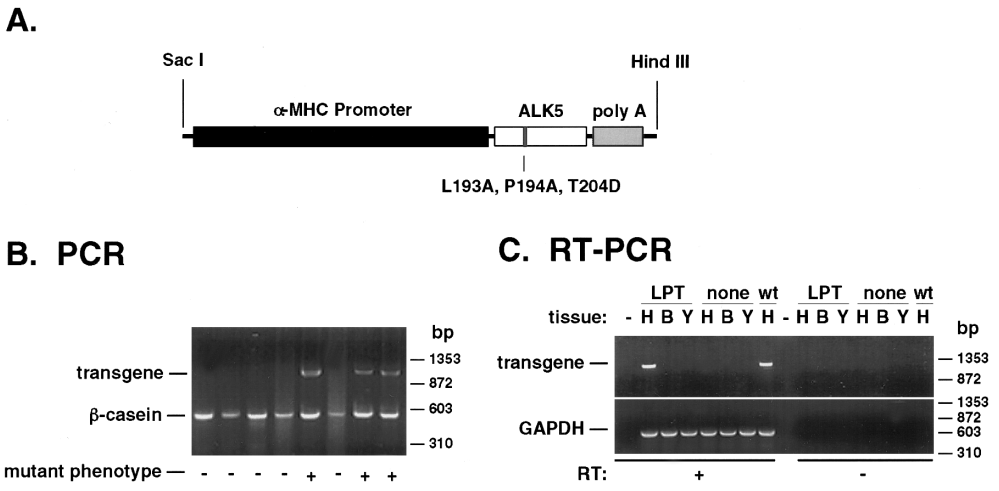

FIG. 1. Expression of activated ALK5 in embryonic myocardium. (A) The αMHC-ALK5^{L193A,P194A,T204D} vector. (B) Representative PCR screening of 8 littermates at E10.5. (C) Expression and cardiac specificity of the transgene were corroborated by RT–PCR. No product was detected in the absence of reverse transcriptase (RT). LPT, the αMHC-ALK5^{L193A,P194A,T204D} transgene; none, transgene-negative littermate; wt, αMHC-wild-type ALK5 transgene; H, heart; B, body; Y, yolk sac.

from two to four pups per recipient. By comparison with the 25% rate for transgene-positive founders receiving wild-type ALK5, only one weanling mouse ever was found with activated ALK5 (5%; $P = 0.026$ by χ^2 test; Table 1). This mouse, in turn, was unable to produce transgene-positive progeny, despite repeated mating.

To test the hypothesis that directing activated ALK5 to myocardium caused embryonic lethality, and did so via altered cardiac development, mouse embryos were harvested at E9.5 and E10.5 and genotyped by the polymerase chain reaction (PCR; Fig. 1B; Table 1). Despite the virtual absence of transgene-positive mice at weaning and even E18, the prevalence of activated ALK5-positive embryos was 27% at E10.5 and 21% at E9.5, comparable to that for wild-type ALK5. In agreement with the expression of endogenous αMHC as early as E7.5–8.0 (Lyons *et al.*, 1990), transgene expression was substantiated in myocardium at E10.5, and was not detected in the body or yolk sac by reverse transcriptase–PCR (RT–PCR; Fig. 1C).

The Cardiac-Specific, Activated ALK5 Gene Arrests Loopingenesis

Whereas looping morphogenesis ordinarily is complete by E10.5, the hearts of activated ALK5 mice remained linear and were dilated at this age, surrounded by a distended pericardium and pericardial effusion; growth retardation was marked (Fig. 2A). Necrosis was evident in embryos at later time points (not shown). Growth retardation and developmental arrest were evident even by E9.5 (Fig. 2B), roughly comparable to normal embryos at E8.5. At E9.5, activated ALK5 embryos contained 4 somites and had not yet undergone turning morphogenesis; transgene-negative littermates had 15 somites and had completed turning.

TABLE 1
Early Lethality of the αMHC-ALK5$^{L193A,\ P194A,\ T204D}$ Transgene

Age	Recipient mothers	Total embryos	Mean litter size	Transgene (+) embryos	Phenotype (+) embryos
αMHC-ALK5$^{L193A,\ P194A,\ T204D}$					
Weaned mice	7	20	2.8	1 (5%)	0 (0%)
E18.5 days	2	9	4.5	1 (11%)	0 (0%)
E10.5 days	13	101	7.8	27 (27%)	21 (78%)
E9.5 days	8	47	5.9	10 (21%)	8 (80%)
αMHC-ALK5					
Weaned mice	4	24	6	9 (25%)	0 (0%)
E10.5 days	2	23	11.5	5 (22%)	0 (0%)

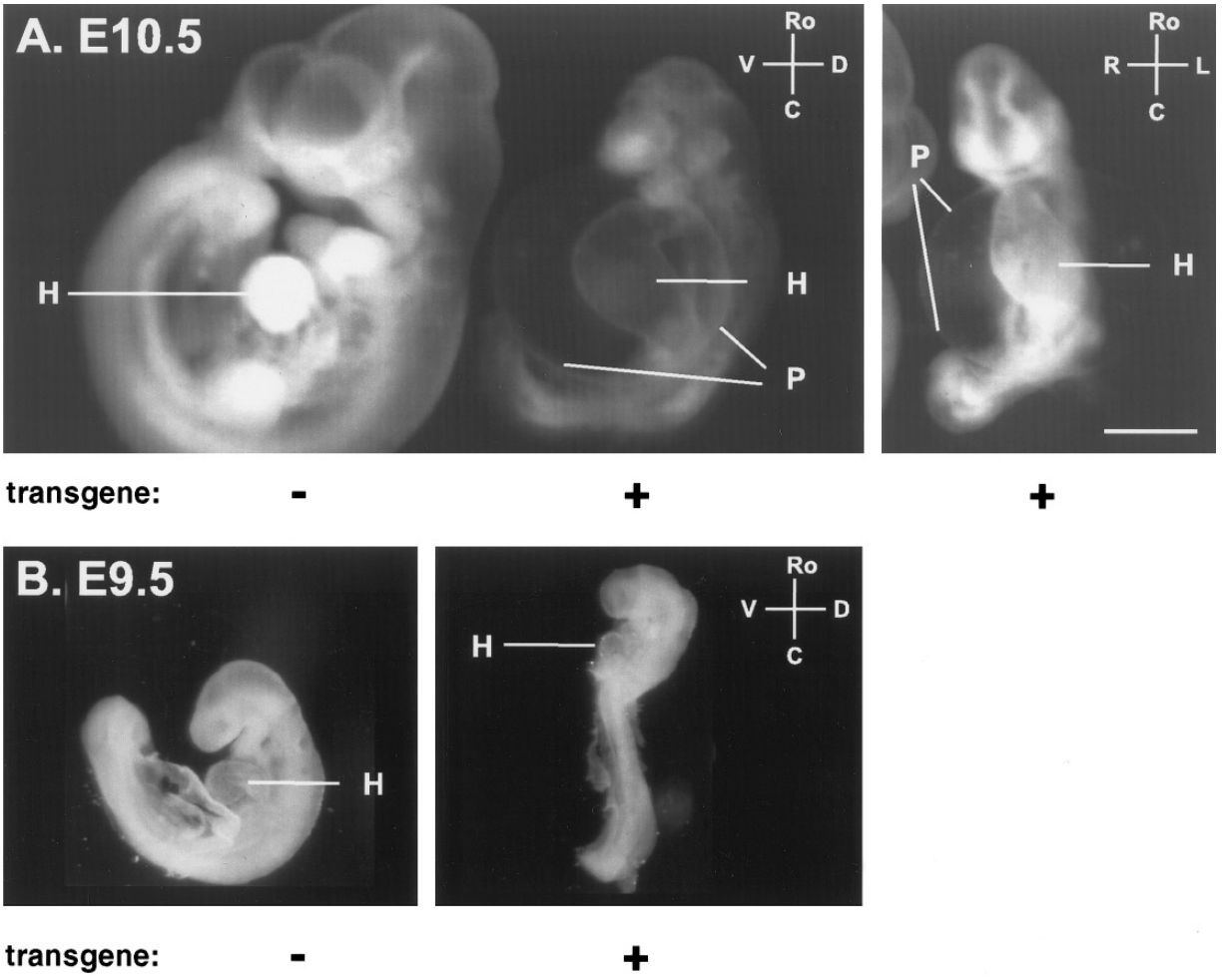

FIG. 2. Morphology of transgene-positive and -negative littermates at E10.5 (A) and E9.5 (B). The block to looping morphogenesis was associated specifically with activated ALK5 and was never observed with the wild-type transgene. Bar, 1 mm. Lateral views are shown, except for the frontal view at E10.5 (upper right). D, dorsal; V, ventral; R, right; L, left; Ro, rostral; C, caudal; H, heart; P, pericardium.

Because each embryo results from an independent injection, insertional mutagenesis cannot account for this phenotype. Growth and development were not grossly affected by wild-type ALK5, using the identical vector.

In transgene-negative littermates at E10.5, immunostaining for sarcomeric MHC delineated the four cardiac chambers as normally developed (Fig. 3C). The left and right ventricles were separated by an incomplete interventricular septum, appropriate to this age, and both contained trabeculated myocardium. In activated $ALK5^{L193A,P194A,T204D}$ embryos at E10.5, the heart consisted of an amorphous, poorly delineated chamber, consistent with the dilated linear heart tube, with poor trabeculation (Fig. 3D). Taken together, cardiac dilatation, lack of chamber demarcation, pericardial effusion, and hypocellularity strongly suggest embryonic heart failure as the cause of death, as in other mutations disrupting cardiac morphogenesis (Lin *et al.*, 1997; Lyons *et al.*, 1995; Srivastava *et al.*, 1997; Sucov *et al.*, 1994).

Activated ALK5 Does Not Prevent Cardiac Myocyte Differentiation

The anatomy of activated ALK5 embryos was especially similar to the linear, dilated heart tube in homozygous Nkx $2.5^{-/-}$ embryos (Lyons *et al.*, 1995). However, no change was seen in Nkx 2.5 expression, by *in situ* hybridization (Figs. 3A and 3B). Whereas MLC_{2V} was absent in mice lacking Nkx2.5 (Lyons *et al.*, 1995), MLC_{2V} was expressed despite the presence of activated ALK5, with levels and spatial distribution similar to wild-type mice at E8.5 (Figs. 3E and 3F); normally, MLC_{2v} is confined to the future ventricle by E9.0 (O'Brien *et al.*, 1993). Expression of dHAND also was comparable to that in developmentally

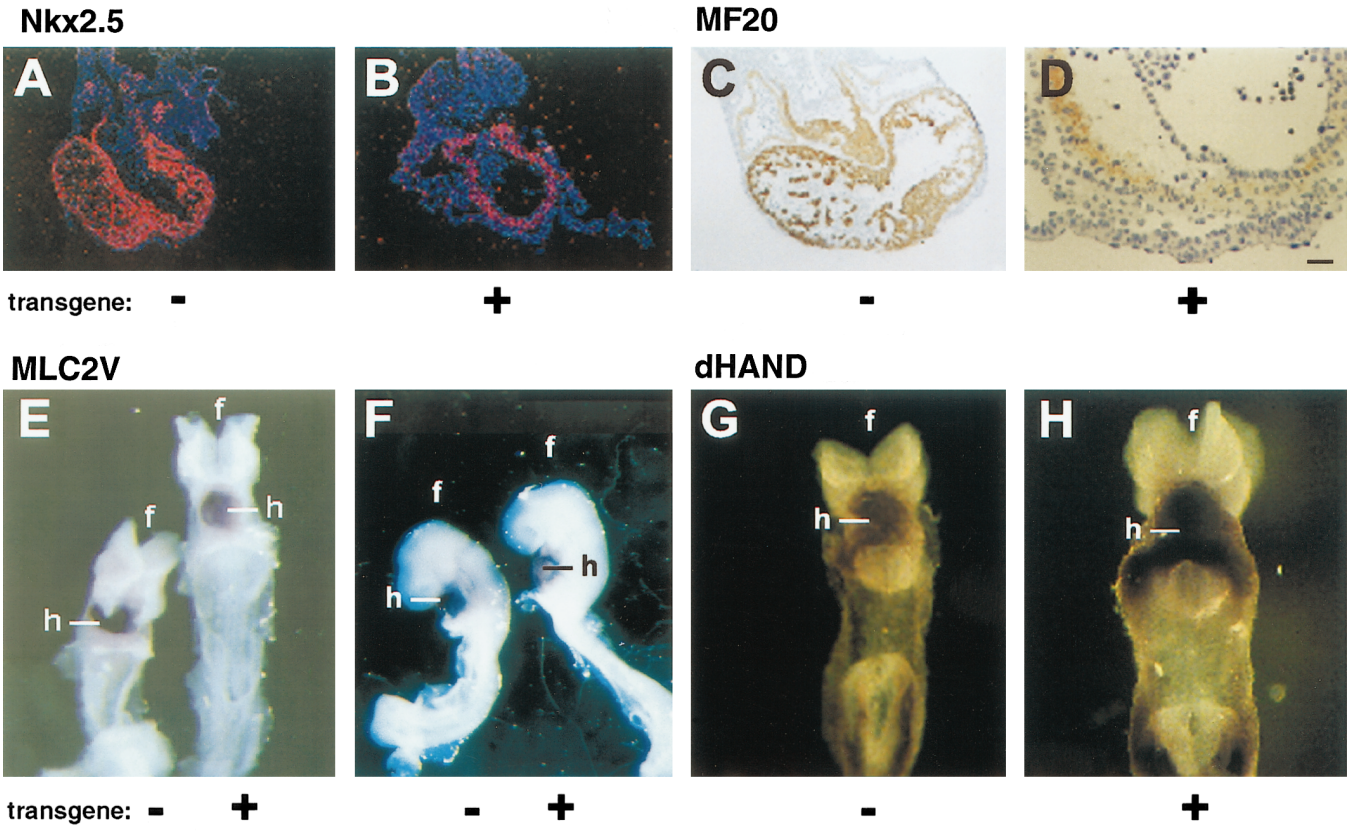

FIG. 3. Activated ALK5 does not prevent cardiac myocyte differentiation. Mutant embryos, E9.5; wild-type embryos, E8.5 (matched for developmental stage). (A, B) *In situ* hybridization for Nkx2.5. (C, D) Immunoperoxidase staining for sarcomeric MHC. (E–H) Whole-mount *in situ* hybridization for (E, F) MLC_{2v} and (G, H) dHAND. E and F are frontal and side views of the same embryos. Bar: A–C, 100 μm; D, 25 μm; E and F, 1 mm; G and H, 750 μm. h, heart; f, neural fold.

matched control mice (E8.5; Figs. 3G and 3H). Thus, no obvious defect was seen in expression of cardiogenic factors whose absence produces a similar structural phenotype.

Activated ALK5 Induces Precocious Expression of p21 in Embryonic Myocardium

To explore an alternative mechanistic basis for the myocardial defects, cardiac RNA was analyzed by RT–PCR for altered expression of TGFβ-responsive growth control proteins. Cell cycle arrest by TGFβ involves regulation of the cyclin-dependent kinase (Cdk)/pocket protein/E2F pathway via at least two mechanisms—induction of Cdk inhibitors including p21 (Reynisdottir *et al.*, 1995) and down-regulation of E2F-1 (Li *et al.*, 1997). RT–PCR was performed in the linear range for template cDNA, using cardiac RNA from E10.5 embryos (Fig. 4). No change was seen in GAPDH (a constitutive control), or in the cardiac-restricted genes, αMHC and MLC_{2V}. By contrast, E2F-1 was reduced fourfold and p21 markedly up-regulated in the mutant hearts. Activated ALK5 did not grossly alter extracellular matrix deposition in myocardium, as assessed by Masson's trichrome stain and immunostaining for type I collagen, and did not induce cardiac apoptosis, as assessed by *in situ* nick end-labeling (not shown).

DISCUSSION

Constitutive Signaling by ALK5 Perturbs Looping Morphogenesis

Thus, activated ALK5, directed to the myocardium, arrests cardiac-looping morphogenesis in mice. Developmental regulation of the αMHC promoter suggests that αMHC-driven transgenes would be expressed in the ventricle predominantly after birth (Subramaniam *et al.*, 1991). However, the full-length, wild-type αMHC promoter is sufficiently functional in midgestation embryos for activated ALK5 to disrupt cardiac organogenesis, with defective looping, impaired chamber demarcation, hypocellularity, dilatation, and embryonic lethality. These morphological features especially resemble those of null mutations for

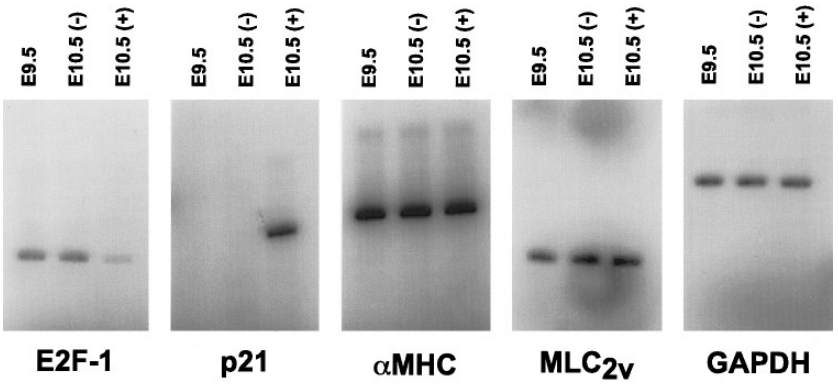

FIG. 4. RT–PCR. p21 was markedly up-regulated by activated ALK5, whereas E2F-1 was reduced at least fourfold. Transgene-positive embryos were studied at E10.5 (+). Transgene-negative littermates are shown at E10.5 (−) with additional control animals, for comparison, at E9.5. No change was observed in αMHC, MLC_{2V}, or GAPDH.

Nkx2.5 (Lyons *et al.*, 1995) and N-cadherin (Radice *et al.*, 1997), which both cause death around E10.5, marked by growth retardation, pericardial distension, and a linear heart tube with varying lateral asymmetry. A related phenotype is noted in knockouts of dHAND (Srivastava *et al.*, 1997) and MEF2C (Lin *et al.*, 1997). Conversely, at least in *Xenopus*, overexpression of Nkx2.5 can augment heart size, perhaps by increased recruitment of cells at the margin of the cardiogenic field (Chen and Fishman, 1996).

In principle, therefore, any of these genes might operate downstream (or upstream) from ALK5. However, activated ALK5 did not cause myocardial cell disaggregation, as in N-cadherin$^{-/-}$ mice (Radice *et al.*, 1997), or marked atrial enlargement, as in mice lacking MEF2C (Lin *et al.*, 1997). Moreover, no defect was seen in the expression of Nkx2.5 itself, in MLC_{2V}, which is absent from Nkx2.5$^{-/-}$ embryos (Lyons *et al.*, 1995), or in dHAND and αMHC, which both are deficient in MEF2C$^{-/-}$ mice (Lin *et al.*, 1997). Thus, the phenotype evoked by activated ALK5 does not suggest interference with N-cadherin or these cardiogenic factors. Hypocellular myocardium also is prominent in mice lacking N-myc, RXRα, TEF-1, β-adrenergic receptor kinase-1, and neuregulin or its receptors, Erb2 and Erb4 (Chen *et al.*, 1994; Jaber *et al.*, 1996; Marchionni, 1995; Sucov *et al.*, 1994). However, looping morphogenesis was not disrupted in any of these seven deficiencies. Hence, normal cardiac looping appears independent of growth per se, though the latter proteins might become critical only after looping has occurred.

The TGFβ Superfamily and Cardiac Looping

These results, using a constitutively activated receptor, are consistent with models proposed for left–right specification by asymmetric localization of a TGFβ family member (Chen *et al.*, 1997a; Hyatt *et al.*, 1996; Levin *et al.*, 1995; Lowe *et al.*, 1996; Meno *et al.*, 1996; Supp *et al.*, 1997). Two especially recent lines of evidence which strengthen this inference are the predominant abnormalities of cardiac BMP4 expression in left–right asymmetry mutants identified through large-scale screening of zebrafish (Chen *et al.*, 1997a), together with the existence of mutations in nodal itself in humans with abnormal situs (Gebbia *et al.*, 1997). As an illustration that looping can be altered via a gain-of-function mutation, the zebrafish laterality mutant *dino* causes expanded expression of BMP4, is mimicked by overexpression of BMP4, and is partially rescued by inhibitors of BMP signaling (Hammerschmidt *et al.*, 1996). Our findings therefore suggest the provisional interpretation that signaling by ALK5 might override endogenous asymmetric cues for left–right patterning of the heart tube. Given the expected timing for αMHC induction in the linear heart tube (Lyons *et al.*, 1990), it is perhaps more likely that this transgene interferes, instead, with the resulting asymmetric effector pathways. Because constitutive signaling by ALK5 induced p21, a Cdk inhibitor associated with terminal differentiation in muscle (Parker *et al.*, 1995), it would be intriguing to learn if p21 is necessary or sufficient for the block to looping here. Although the available information is insufficient to draw the conclusion that asymmetric cell cycle control is a mechanism for asymmetric cardiac morphogenesis, recent findings in chick embryos support the general conclusion that cardiac morphogenesis is mediated, in part, by developmental regulation of cell cycle inhibitors, as shown by adenoviral gene transfer to confer precocious expression of the homeodomain protein, Gax (Fisher *et al.*, 1997).

Activated ALK5 exhibits fidelity to the actions of exogenous TGFβ (Charng *et al.*, 1996; Wieser *et al.*, 1995), but we do not dismiss the possibility that activated ALK5 might simulate proteins distinct from TGFβ itself. First, ALK5 differs from most type I receptors but closely resembles the activin receptor IB in structure and signaling function (Carcamo *et al.*, 1994). Second, the responsible receptors have not yet been ascertained either for nodal or for lefty, two members of the TGFβsuperfamily which are

most directly implicated in left–right asymmetry in mice. The impairment of left–right patterning shown in mice lacking the activin IIB receptor may be instructive in this regard (Oh and Li, 1997): as also was true for an earlier deletion of the activin IIA receptor (Matzuk *et al.*, 1995), the IIB-deficient mice do not resemble the knockouts of activin subunits themselves, singly or in combination. Thus, ligands in addition to activin must signal via these receptors during embryogenesis. By analogy, the ALK5 phenotype here may conceivably point to a role for family members distinct from TGFβ1-3.

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